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Uridine Diphosphate Glucuronic Acid as Glucuronyl Donor in the Synthesis of 'Ester', Aliphatic and Steroid Glucuronides

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There are two types of glucuronide, depending on the aglycone: alcohols and phenols give rise to glycosidic or 'ether'-linked glucuronides, and certain carboxylic acids to acylal or 'ester'-linked conjugates (see Teague, 1954).

It is known that uridine diphosphate glucuronic acid (UDPglucuronic acid) acts as a glucuronyl donor in the synthesis of 'ether' glucuronides, transferring its glucuronic acid to an acceptor (R.OH), an 'ether' glucuronide and uridine 5'-pyrophosphate (UDP) being formed in the following manner (Dutton & Storey, 1953, 1954; Storey & Dutton, 1955; Isselbacher & Axelrod, 1955):

R.OH + UDPglucuronic acid → R.O.glucuronic acid + UDP.

Storey & Dutton (1955) suggested that UDP-glucuronic acid is an 'active' form of glucuronic acid, taking part in the general tissue metabolism of that substance; it therefore seemed of interest to study the range of compounds acting as acceptors in the above reaction. The aglycones previously known to be conjugated in this manner were *o*-aminophenol, (−)-menthol (Dutton & Storey, 1951), phenolphthalein, thyroxine and some corticosteroids (Isselbacher & Axelrod, 1955), all yielding 'ether' glucuronides.

The present work extends these observations to include as acceptors two aromatic acids forming 'ester' glucuronides and, as examples of aliphatic compounds, an acid and an alcohol giving rise to an 'ester' and an 'ether' type of conjugate respectively. Evidence for the formation of the glucuronides of pregnane-3α:20α-diol and of some other compounds is also presented.

Of the aglycones chosen, *p*-aminobenzoic acid has been isolated as its 'ester' glucuronide from rabbit urine (Bray, Lake, Neale, Thorpe & Wood, 1948); formation of *o*-aminobenzoyl glucuronide has been reported in rat-liver slices (Shirai & Ohkubo, 1954*a*); 1:1-*dimethylpropanol* (*tert.*-amyl alcohol) and α-ethylhexanoic acid are excreted as 'ether' and 'ester' glucuronides respectively in rabbit urine (Kamil, Smith & Williams, 1953*a*, *b*). Pregnane-3α:20α-diol and 3α-hydroxyandrostan-17-one (androsterone) glucuronides have been isolated from human urine (Venning & Browne, 1936; Edwards & Kellie, 1956), and 4:4'-*dihydroxy*-αβ-diethylstilbene (stilboestrol) glucuronide from rabbit urine (Mazur & Shorr, 1942); *allo*pregnane-3β:20β-diol glucuronide does not yet appear to have been detected, although the 3α:20α-isomer has been found in human urine, conjugated with an unidentified substance (Kyle & Marrian, 1951).

Preliminary reports of this work have already appeared (Dutton, 1955; Storey & Dutton, 1956; Dutton & Spencer, 1956).

EXPERIMENTAL

Materials

Acceptor substrates and reference compounds. *o*-Aminobenzoic (OAB) and *p*-aminobenzoic (PAB) acids were commercial samples thrice recrystallized from water.

p-Aminobenzoyl glucuronide (PABG) was crystallized from an ether-extracted soln. of an equimolecular complex of PAB and PABG, and also recrystallized twice from a cruder PABG specimen (original materials were 9 years old and kindly given by Dr W. V. Thorpe). Potassium β-2-ethylhexanoyl-D-glucuronide monohydrate was recrystallized from a cruder specimen presented by Professor R. T. Williams, who also generously sent a sample of tri-*o*-acetyl-β-1:1-dimethylpropyl-D-glucuronide methyl ester; from this, a gum presumed to be the potassium salt of the free glucuronide was prepared by the gradual addition of the calculated amount of ethanolic KOH at 20° (method kindly supplied by Professor R. T. Williams). α-Ethylhexanoic acid, b.p. 220–222°, was prepared by the oxidation of commercial 2-ethyl-*n*-hexanol by method (*a*) of Kenyon & Platt (1939). Pregnane-3α:20α-diol was generously presented by Dr J. K. Grant, and sodium pregnanediol glucuronidate was a specimen from the Medical Research Council steroid collection. *allo*Pregnane-3β:20β-diol, androsterone and stilboestrol were commercial samples (Light and Co.).

UDPglucuronic acid. In preliminary experiments, either the 'crude' or barium–ethanol fractionated nucleotide was used: for confirmation, chromatographically pure preparations were employed (Storey & Dutton, 1955). Glucuronic acid from the nucleotide did not interfere with estimations of conjugate glucuronic acid.

β-Glucuronidase. This was prepared by the method of Kerr, Graham & Levvy (1948). Incubation for hydrolysis was at pH 5·1 for 3 hr. at 37°.

Homogenate

Tissue dispersion. The method was that used previously (Dutton & Storey, 1954). With aliphatic and steroid substrates a 20% (w/v) mouse-liver homogenate replaced the 10% concn. normally employed.

Fractionation of homogenate. Fractionation followed the method of Schneider & Hogeboom (1950); all operations were done at 0°. A 10% (w/v) mouse-liver homogenate (10 ml.) was prepared in 0·25 M sucrose; the nuclei were centrifuged down at 700 *g* for 10 min., the mitochondria at 5000 *g* for 20 min., and the microsomes at about 21 000 *g* for 1½ hr.; the 'high-speed supernatant' remained after such centrifuging. All particulate fractions were washed by resuspension in sucrose, followed by a further centrifuging, and were finally suspended in 9 ml. of sucrose soln. (In preliminary experiments, 1 ml. of M-KCl was added to the nucleus- and mitochondrion-free homogenate, and the aggregated microsomes were centrifuged down at about 21 000 *g* for 40 min., being then washed in isotonic KCl and resuspended in sucrose.) Excess of sucrose interfered in glucuronic acid estimation of the aliphatic conjugates; in such cases the washed particles were resuspended in isotonic KCl, the 'high-speed supernatant' being that resulting from the centrifuging (at about 21 000 *g* for 40 min.) of a homogenate dispersed in isotonic KCl.

Reaction medium

This was essentially that used for *o*-aminophenyl glucuronide synthesis (Dutton & Storey, 1954; Storey & Dutton, 1955). Pure UDPglucuronic acid when present was of final concn. 0·05 mM, measured from the absorption at 260 mμ. (see Storey & Dutton, 1955).

OAB and PAB as substrates. The *o*-aminophenol–ascorbic acid mixture was replaced in the small-scale reaction medium of Storey & Dutton (1955) by 0·14 mM OAB or PAB (as the potassium salt). The digest (0·6 ml. total volume) was incubated for 30 min. at 37°.

α-Ethylhexanoic acid and tert.-amyl alcohol as substrates. α-Ethylhexanoic acid (as the potassium salt) and *tert.*-amyl alcohol were 1·38 and 14·2 mM respectively in a total volume of 2 ml. or 0·6 ml. of medium in suitable stoppered flasks.

Androsterone, stilboestrol, allopregnanediol and pregnanediol as substrates. Because of the insolubility of these substrates and the difficulty of achieving their uniform dispersion, it is not possible to record their final molarities in the medium. They were usually added in great excess (2 mg./ml.) to the homogenizing soln. and dispersed through the disrupting tissue. Occasionally they were homogenized in fresh human serum before addition to the medium, but this tended to increase control readings in the naphtharesorcinol reaction. To achieve measurable conjugation, the total volume of medium was increased to 20 ml./flask, with a 20% (w/v) homogenate, and incubation was for 1 hr. at 37°.

Estimation of conjugates

OAB and PAB as substrates. A modification of the method of Shirai & Ohkubo (1954*a*) was used. After incubation, 1·4 ml. of water and 0·4 ml. of M-trichloroacetic acid were added, and protein was centrifuged down. To a 1·6 ml. portion of the supernatant, 0·28 ml. of 0·5 M-Na_2CO_3 and 0·12 ml. of water were added (bringing the pH to 4·0), and the soln. was extracted thrice with 3 ml. portions of ether to remove free substrate. The aqueous soln. was then treated with 0·4 ml. of N-KOH and heated at 55° for 1 hr. to hydrolyse the 'ester'-linked conjugate, before being brought to pH 4 with N-HCl and again extracted thrice with 3 ml. portions of ether. The ethereal layer contained the acid aglycone released from 'ester' conjugation by hydrolysis and was itself extracted twice with 0·8 ml. volumes of 0·1 N-NaOH. To the aqueous phase from this extraction was added 0·06 ml. of 4 N-HCl, followed by 0·1 ml. each of 0·1% $NaNO_2$, 0·5% ammonium sulphamate, 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride. After incubation at 37° for 3 hr., the extinctions were read in the 1 cm. microcells of the Spekker absorptiometer, with Ilford filter no. 605 (peak transmission, 550 mμ.).

α-Ethylhexanoic acid and tert.-amyl alcohol as substrates. After incubation, proteins were precipitated by an equal volume of 3% (w/v) sulphosalicylic acid, and centrifuged down. With α-ethylhexanoic acid as substrate, 3 ml. portions of the supernatant were extracted once with 2 vol., and twice with 1 vol. of ethyl acetate. The organic phase was taken to dryness at 40° in a current of air. With *tert.*-amyl alcohol as substrate, 3 ml. portions of the supernatant were saturated with $(NH_4)_2SO_4$ and extracted thrice with equal volumes of a 1:4 (v/v) ethanol–ether mixture (Kamil *et al.* 1953*a*); the organic phase was then taken to dryness.

To the resulting gums, 3 ml. of water, 2 ml. of 18 N-H_2SO_4 and 2 ml. of fresh 0·35% (w/v) aqueous naphtharesorcinol soln. were added, and the whole was heated at 100° for 1 hr., cooled, and extracted with a mixture of 4 ml. of ethanol and 6 ml. of toluene. The extinction of the organic phase was read in the Spekker absorptiometer, with Ilford filter no. 605.

Androsterone, stilboestrol and allopregnanediol as substrates. Protein was removed by heat coagulation and centrifuging, and the conjugates were extracted from the acidified (HCl) supernatant five times with equal volumes of

ethyl acetate, or thrice with one-half volume of *n*-butanol. The solvent was then removed, if necessary *in vacuo*; the naphtharesorcinol reaction was performed as above on the resulting gum.

Paper chromatography

This was carried out by the ascending or ascending–descending method (Block, 1950) in all-glass tanks, with Whatman no. 541 paper; unless otherwise stated the temp. was 20°. Spots were identified by reference to standard compounds rather than by comparison with published R_F values.

OAB, PAB, o-aminophenol and their glucuronides. Solvents used were (*a*) *n*-butanol–acetic acid–water (4:1:1, by vol.) (Shirai & Ohkubo, 1954*a*); (*b*) *n*-butanol–1·5 N-NH_3 soln. (1:1, v/v) (Brown, 1950); (*c*) ethanol–M ammonium acetate, pH 7·0 (3:2, v/v). Chromatograms were sprayed lightly with 0·1% $NaNO_2$ freshly prepared in 0·1 N-HCl, dried in air, and sprayed again with 0·2% ethanolic *N*-(1-naphthyl)ethylenediamine dihydrochloride. *o*-Aminophenol gave a yellow, not purple, spot. The naphtharesorcinol test for glucuronic acid was performed on eluates of chromatogram areas.

α-Ethylhexanoic acid. Solvents used were (*a*) *n*-butanol–1·5 N-NH_3 soln. (1:1, v/v) (Brown, 1950); (*b*) *iso*octane–acetone–ethanol (95%)–conc. NH_3 soln. (40:30:30:1, by vol.) (Nair, 1953). For (*a*) the spray was bromothymol blue and for (*b*) bromophenol blue.

tert.-*Amyl alcohol.* The xanthogenate was chromatographed in *n*-butanol saturated with 2% (w/v) aqueous KOH (see Kariyone & Hashimoto, 1951), and the chromatograms were examined by u.v. light (365–366 mμ.). On heating the paper, the brown fluorescence of the xanthogenate was replaced by a more easily seen blue fluorescence.

2-*Ethylhexanoyl and dimethylpropyl glucuronides.* The solvent was the *n*-butanol–1·5 N-NH_3 soln. mixture used above.

Pregnanediol. Toluene was the mobile phase, on paper strips previously impregnated with propylene glycol, the spray being $SbCl_3$ in nitrobenzene [claimed by Rosenkrantz (1953) to be more sensitive towards hydroxylated steroids] or in $CHCl_3$. Subsequent heating yielded pale-brown spots with a red-brown fluorescence in u.v. light.

RESULTS

Synthesis of conjugates

Conjugates which behaved in the estimation procedures like the corresponding glucuronides of the substrates were formed only when UDPglucuronic acid was present during incubation of the complete reaction mixture; control flasks (where substrate and UDPglucuronic acid respectively were added after incubation) were always run. It remained to confirm that these conjugates were those of the substrates with glucuronic acid.

o- *and* p-*Aminobenzoic acid as substrates*

Evidence for the aglycone. The aglycone of both conjugates behaved as if it were 'ester'-linked, being completely hydrolysed under the mild alkaline conditions of the estimation. According to Shirai & Ohkubo (1954*a*), aniline, acetanilide, 5-hydroxyanthranilic acid and *N*-acetylanthranilic acid do not interfere in this estimation. Added *o*-aminophenyl glucuronide was not hydrolysed, so that any diazotizable 'ether' glucuronide resulting from substrate breakdown should not have been measured; only a trace of unhydrolysed diazotizable material was ever detected. After such mild hydrolysis the liberated aglycones were extracted with ether, concentrated, and chromatographed alongside known OAB, PAB and *o*-aminophenol. R_F values (which for these and subsequent chromatograms are detailed in Table 1) confirmed that the aglycones were the respective original substrates.

Evidence for the glucuronic acid moiety. The conjugates were synthesized on a large scale, by using 30 ml. reaction mixtures incubated for 1 hr. After 3 min. at 100°, the solutions were centrifuged, brought to pH 4, and each supernatant was extracted thrice with 45 ml. portions of ether to remove free substrate. The aqueous phase was neutralized and concentrated to about 1 ml. *in vacuo*. The PAB conjugate travelled on paper chromatograms exactly as did known PABG (see Table 1).

The conjugates could not be separated satisfactorily from the nucleotide glucuronic acid by extraction; the naphtharesorcinol reaction, however, when performed on eluates of areas on the above chromatogram indicated a positive result at the level of the conjugate as well as at the glucuronic acid and UDPglucuronic acid levels (see Table 1).

Confirmatory evidence came from the use of β-glucuronidase. As also reported by Levvy & Worgan (1955), it was found that this enzyme hydrolysed 'ester' glucuronides (here PABG) with strong specific inhibition from boiled saccharate solution (see Levvy, 1952). Conjugates from large-scale synthesis were treated with the enzyme, with and without the inhibitor. Ether-soluble compounds were released, behaving like the free aglycones both in colour estimations and on chromatography; such liberation was strongly inhibited by boiled saccharate solution (see Table 2). Since esterase activity of the enzyme is limited to 'ester' glucuronides (Levvy & Worgan, 1955), the aglycones must have been conjugated with glucuronic acid.

α-*Ethylhexanoic acid as substrate*

Evidence for the aglycone. The area on the conjugate chromatogram giving a positive naphtharesorcinol reaction (see below) was eluted. One portion of the eluate (*A*) was hydrolysed with 0·3 N-HCl at 100° for 15 min. (Kamil *et al.* 1953*b*) and extracted twice with an equal volume of ether; 1·0 ml. of M N-NaOH was added to the ethereal layer, the ether removed by aeration and the solution

neutralized and concentrated. A second portion (*B*) was treated similarly, but without hydrolysis, as a control for free substrate. The two concentrates were then chromatographed beside known α-ethylhexanoic, valeric and propionic acids. *A* gave rise to a spot travelling like known α-ethylhexanoic acid (see Table 1); *B* yielded no detectable spot.

Evidence for the glucuronic acid moiety. The conjugate was synthesized on a large scale, heat-coagulated protein centrifuged down, and the supernatant brought to pH 3 with concentrated HCl before extraction with ethyl acetate. The concentrated extract was then chromatographed as a band alongside known 2-ethylhexanoyl glucuronide. Strips from this chromatogram were subjected to the naphtharesorcinol reaction; a positive result was obtained at the level of known 2-ethylhexanoyl glucuronide (see Table 1).

β-Glucuronidase again provided confirmatory evidence, hydrolysing the conjugate markedly unless boiled saccharate solution was present (see Table 2).

Table 1. *Paper chromatography of unknown conjugates and their aglycones*

Solvents employed were: (i) *n*-butanol–acetic acid–water; (ii) *n*-butanol–1·5 N-NH_3 soln.; (iii) ethanol–M ammonium acetate; (iv) *iso*octane–acetone–ethanol–conc. NH_3 soln.; (v) *n*-butanol saturated with 2% aqueous KOH. Details are given in the Experimental section. All chromatograms were run at 20°, by ascending or ascending–descending method. Compounds in parentheses were substrates in reactions whereby respective unknown conjugates were formed. In this and subsequent tables — means 'not determined'.

Compound	R_F mean values with solvents (i)	(ii)	(iii)	(iv)	(v)
o-Aminobenzoic acid	0·93	0·34	—	—	—
Aglycone from conjugate (*o*-aminobenzoic acid)	0·92	0·34	—	—	—
p-Aminobenzoic acid	0·88	0·14	—	—	—
Aglycone from conjugate (*p*-aminobenzoic acid)	0·87	0·15	—	—	—
o-Aminophenol	0·72	—	—	—	—
p-Aminobenzoyl glucuronide	0·34	—	0·70	—	—
Conjugate (*p*-aminobenzoic acid)	0·32	—	0·70	—	—
Conjugate (*o*-aminobenzoic acid)	0·38	—	—	—	—
o-Aminophenyl glucuronide	0·23	—	—	—	—
Uridine diphosphate glucuronic acid	—	—	0·12	—	—
Glucuronic acid	—	—	0·35*	—	—
α-Ethylhexanoic acid	—	0·70	—	0·75	—
Aglycone from conjugate (α-ethylhexanoic acid)	—	0·72	—	0·75	—
Propionic acid	—	0·15	—	—	—
Valeric acid	—	0·32	—	—	—
tert.-Amyl alcohol (as xanthogenate)	—	—	—	—	0·24*
Aglycone from conjugate (*tert.*-amyl alcohol) (as xanthogenate)	—	—	—	—	0·24*
2-Ethylhexanoyl glucuronide	—	0·38*	—	—	—
Conjugate (α-ethylhexanoic acid)	—	0·38*	—	—	—
tert.-Amyl alcohol glucuronide	—	0·11*	—	—	—
Conjugate (*tert.*-amyl alcohol)	—	0·11*	—	—	—

* Indicates smearing of the spot.

Table 2. *Hydrolysis of conjugates by β-glucuronidase*

All flasks contained 0·06 ml. of 0·3M citrate buffer, pH 5·1; where indicated, also 0·08 ml. of β-glucuronidase and boiled saccharate soln. Total vol. was 0·6 ml. and incubation was for 3 hr. at 37°. *A*?, *B*?, *C*? represent conjugates obtained with *p*-aminobenzoic acid, α-ethylhexanoic acid and *tert.*-amyl alcohol as substrates, respectively; *A*, *B*, *C* are the respective authentic glucuronides. Final molarity of saccharate is quoted.

β-Glucuronidase	Saccharate (mM)	Wt. in conjugate (μg.) *p*-Aminobenzoic acid *A*?	*A*	Glucuronic acid *B*?	*B*	*C*?	*C*
(i) Not present	0	2·0	2·5	3·5	6·1	1·4	3·7
(ii) Present	0	1·2	1·8	1·9	4·3	0·8	2·6
(iii) Present	0·12	1·9	2·4	—	—	—	—
(iv) Present	0·50	—	—	3·1	5·9	1·2	3·4
Percentage hydrolysis in (ii)		40	28	46	31	43	30
Percentage hydrolysis in (iii) or (iv)		5	4	11	3	14	8
Percentage inhibition of hydrolysis in (ii) by:							
0·12 mM saccharate		88	85	—	—	—	—
0·50 mM saccharate		—	—	75	89	67	73

tert.-Amyl alcohol as substrate

Evidence for the aglycone. Work with the aglycone proved difficult. That chromatogram area giving a positive naphtharesorcinol reaction (see below) was eluted, washed twice with an equal volume of ether to remove any free substrate, hydrolysed with β-glucuronidase and again extracted twice with an equal volume of ether. The concentrated ether layer was then treated to give the alcohol xanthogenate (Kariyone & Hashimoto, 1951), which was chromatographed alongside the xanthogenate of known *tert.*-amyl alcohol. Results were consistent with the aglycone being *tert.*-amyl alcohol (see Table 1).

Evidence for the glucuronic acid moiety. This was obtained from hydrolysis of the conjugate by β-glucuronidase and from inhibition of this process by boiled saccharate solution (see Table 2). On chromatography, the unknown conjugate travelled like *tert.*-amyl alcohol glucuronide (see Table 1).

Steroids and stilboestrol as substrates

Preliminary experiments indicated that when androsterone, stilboestrol and *allo*pregnane-3β:20β-diol were incubated with UDPglucuronic acid in the reaction mixture there was an increase of material extractable at pH 2–3 with ethyl acetate or *n*-butanol and giving a positive naphtharesorcinol reaction (compared with controls where substrates or the nucleotide were added after incubation). Complete extraction of this material was not possible with ethyl acetate; solvents which effected this, such as *n*-butanol, tended also to extract some glucuronic acid originating in the nucleotide. Only slight synthesis was found, some 0·8–3·5 μg. of glucuronic acid being conjugated/700 mg. wet wt. of liver (see Storey & Dutton, 1956), but sufficient to indicate the effect of UDPglucuronic acid. The conjugate obtained with *allo*pregnanediol as substrate was presumably hydrolysed by β-glucuronidase, for after such treatment its glucuronic acid was no longer extractable with *n*-butanol. With pregnane-3α:20α-diol as substrate further confirmation was obtained.

Two 40 ml. volumes of reaction mixture, *A* and *B*, were incubated; pregnanediol was present in *A*, but added to *B* only after incubation. After heat-coagulation of protein and centrifuging, both supernatants were extracted thrice with equal volumes each time of methylene chloride to remove free substrate. The remaining methylene chloride was removed from the aqueous phase *in vacuo*. *A* and *B* were then divided each into two portions; to one portion of each was added boiled saccharate solution of final millimolar concentration. All four solutions were incubated with β-glucuronidase for 5 hr. Protein was removed as formerly, the methylene chloride extractions were repeated, and the extracts concentrated and chromatographed alongside known pregnane-3α:20α-diol. A faint spot at the same level as that of the known substance was observed in the chromatogram of that portion from *A* not treated with inhibitor (see Fig. 1); β-glucuronidase, therefore, liberated from the conjugate a substance travelling like pregnanediol on chromatography in the solvent used and resembling it also in the detection procedure. No trace of a similarly reacting spot was found on the remaining

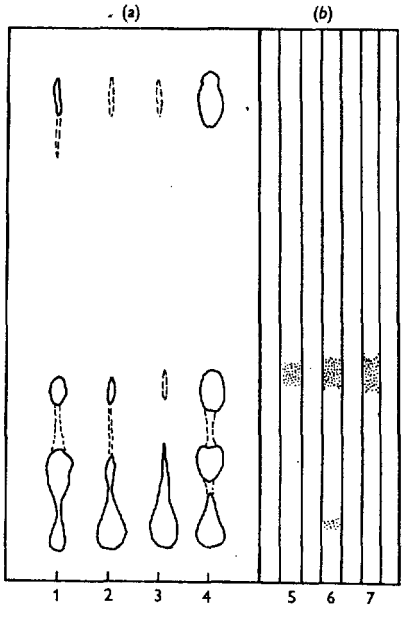

Fig. 1. (*a*) Demonstrating the liberation of uridine 5′-pyrophosphate during the reaction between UDP-glucuronic acid and *p*-aminobenzoic acid. Chromatograms of 1, barium-free soln. of ppt. from reaction mixture treated with barium acetate–ethanol (see text) after 5 min. incubation; 2, as for 1, but with no substrate present; 3, as for 1, but with no incubation; 4, known compounds: from base-line, UDPglucuronic acid, uridine 5′-pyrophosphate, uridine 5′-phosphate, uridine. Solvent system, propan-2-ol–1% aqueous $(NH_4)_2SO_4$ (2:3, v/v). Ultraviolet-absorbing areas are outlined, broken lines indicate faint absorption.

(*b*) Strip chromatograms of 5, compound liberated by β-glucuronidase from conjugate obtained with pregnanediol as substrate; 6, compound liberated by β-glucuronidase from known pregnanediol glucuronide; 7, known pregnanediol. Solvent system, toluene on propylene glycol-impregnated paper. Stippled areas gave red-brown colour and fluorescence after spraying with $SbCl_3$–nitrobenzene (see text). Origins for (*a*) and (*b*) are at the base-line.

three chromatogram strips, though some faint fluorescent smears were common to all four. Mixtures of known pregnanediol and its glucuronide also were treated as above; in the case of the sample treated with *uninhibited* β-glucuronidase, free pregnanediol liberated from the known pregnanediol glucuronide was satisfactorily extracted and detected.

Nature of the reaction

After incubation of PAB for 5 min. with washed microsomes as enzyme source and pure UDPglucuronic acid in the reaction mixture, chromatographic evidence was found for the presence of UDP; some uridine 5'-phosphate (UMP) and a trace of uridine were also present, resulting presumably from UDP breakdown. After the incubation an equal volume of 3% (w/v) sulphosalicylic acid was added, the solution centrifuged and the supernatant extracted twice with half its volume of ether. The aqueous phase was concentrated to 1 ml., adjusted to pH 8–9, and 1 drop of 25% (w/v) barium acetate solution and 1 ml. of ethanol were added; after cooling to 0°, the precipitate was centrifuged down, dissolved in the minimum amount of 0·1 N-HCl and freed of Ba^{2+} ions by precipitation with $(NH_4)_2SO_4$. The supernatant was neutralized, concentrated and chromatographed in propan-2-ol–1% aqueous $(NH_4)_2SO_4$ (3:2, v/v) by the method of Anand, Clark, Hall & Todd (1952). The chromatogram was examined under a Hanovia Chromatolite u.v. light lamp. Controls consisted of UDPglucuronic acid, UDP, UMP, uridine, an identical non-incubated reaction mixture and one containing no substrate; in the last-named, UDP was present only in small amount (see Fig. 1).

Factors influencing synthesis

With OAB, PAB and *o*-aminophenol concentrations of 0·14 mM, 0·7, 0·6 and 0·9 μg. respectively of the aglycone were conjugated after 30 min. incubation. Both the optimum pH (7·4–7·9) and anaerobic nature of 'ester' glucuronide synthesis agreed with that previously found for *o*-aminophenol conjugation (Dutton & Storey, 1954); this held also with microsomes as enzyme source.

Location of enzyme system

The enzyme system responsible for glucuronyl transfer from UDPglucuronic acid to a phenolic acceptor is located in the small-particle, or microsomal, fraction of the homogenate (Dutton & Storey, 1954; Strominger, Kalckar, Axelrod & Maxwell, 1954). After fractionation of the homogenate in 0·25 M sucrose solution by the method of Schneider & Hogeboom (1950), it was found that the microsomal fraction was also the chief site of synthesis of the glucuronides of PAB or of the aliphatic substrates, as illustrated by Table 3. Additions of other fractions slightly increased the synthesis; the Table also shows those combinations of fractions which effected the most synthesis.

Treatment of freeze-dried microsomes with *n*-butanol at −10° (see Morton, 1955) and removal of the solvent *in vacuo*, followed by suspension of the particles in 5 mM phosphate buffer (pH 7·4) and centrifuging at about 21 000 *g* for 60 min., resulted in the release of some 5% of the original enzyme activity into aqueous solution; further work will show if this is a practical method of purification.

Table 3. *Glucuronide synthesis by homogenate fractions*

Each flask received 0·04 ml. of 0·5 M aminotrishydroxymethylmethane buffer (pH 7·4) containing 0·15 M-$MgCl_2$, substrate, 0·05 mM UDPglucuronic acid (final concn.), enzyme source and water to 0·5 ml. *A–L*: 0·14 mM *p*-aminobenzoic acid as substrate. *M–Q*: 14·2 mM *tert.*-amyl alcohol as substrate. Incubated for 30 min. at 37°. Description of fractions in text.

Tissue preparation derived from 10 mg. of whole wet liver	Wt. in conjugate (μg.) *p*-Aminobenzoic acid	Glucuronic acid
(*A*) Total 10% liver–sucrose homogenate (*B* + *C* + *D* + *E*)	1·1	—
(*B*) Nuclear fraction from *A*	0·2	—
(*C*) Mitochondrial fraction from *A*	0·2	—
(*D*) Microsomal fraction from *A*	0·6	—
(*E*) High-speed supernatant from *A*	0	—
(*F*) *B* + *D*; (*G*) *C* + *D*; (*H*) *D* + *E*	0·8; 0·9; 0·7	—
(*I*) *B* + *C* + *D*; (*J*) *B* + *D* + *E*; (*K*) *C* + *D* + *E*; (*L*) *B* + *C* + *E*	0·9; 0·8; 0·7; 0·4	—
(*M*) Total particles from 10% liver–sucrose homogenate (*N* + *O* + *P*)	—	1·9
(*N*) Nuclear fraction from *M*	—	0·3
(*O*) Mitochondrial fraction from *M*	—	0·4
(*P*) Microsomal fraction from *M*	—	0·7
(*Q*) High-speed supernatant from 10% liver–KCl homogenate	—	0

Inhibition of o-aminophenyl glucuronide synthesis

The above results suggested that 'ether' and 'ester' glucuronides, with either aliphatic or aromatic aglycones, were conjugated wholly or partly by the same enzyme system. If this were so, then the substrates studied should act as competitive inhibitors in the *o*-aminophenyl glucuronide-synthesizing system of Dutton & Storey (1954). Such competitive inhibition occurred, with benzoic acid (see Williams, 1947) used in place of PAB or OAB, and with α-ethylhexanoic acid. Table 4 shows, with the relatively crude enzyme preparations available, how inhibition of *o*-aminophenyl glucuronide synthesis varied directly with concentration of competing substrate and indirectly with that of *o*-aminophenol itself.

Presence of enzyme system in tissues other than liver

Although no attempt was made at this time to examine systematically tissues other than liver for 'ester' or aliphatic glucuronide conjugation, mouse kidney and duodenum were studied, following the report by Shirai & Ohkubo (1954*b*) that slices of these organs from the rat synthesized *o*-aminobenzoyl glucuronide (OABG) to an extent comparable with, or (with duodenum) even greater than, that occurring with liver. No glucuronide formation with any of the substrates could be demonstrated by using 20% (w/v) homogenates of saline-washed mouse duodenum. With kidney, however, a variable amount of material behaving like OABG or PABG in the colour estimation was formed; this material, often corresponding in colour density to as much as 30% of the PABG conjugated by a comparable liver homogenate of the same animal, could never be more than 5% hydrolysed by β-glucuronidase. Most of the alkali-labile diazotizable conjugate formed by the kidney with these two substrates must therefore not have been a β-glucuronide. Synthesis by the kidney of glucuronides of other substrates employed was at most 15% of that occurring in liver.

DISCUSSION

The results indicate that *o*- and *p*-aminobenzoyl glucuronides, 2-ethylhexanoyl glucuronide and most probably *tert.*-amyl alcohol glucuronide were synthesized under the above conditions. There is good evidence for the formation of pregnane-3α:2α-diol glucuronide; with the other steroids and stilboestrol, glucuronide formation was evident but in these cases no identification was attempted of the aglycones themselves, which could have been therefore either the original substrates or metabolites thereof.

Chromatographic evidence shows that UDP-glucuronic acid disappears during 'ester' glucuronide formation and UDP appears; this supports the mechanism proposed for 'ether' glucuronide synthesis in the same system (Storey & Dutton, 1955; Isselbacher & Axelrod, 1955) whereby a molecule of glucuronic acid previously linked with UDP is transferred to the acceptor.

It would now appear that UDPglucuronic acid acts as glucuronyl donor to a wide range of aglycones, and that these include alcohols and carboxylic acids of both the aromatic and aliphatic series. Aliphatic glucuronide formation occurs *in vivo* when further oxidation of the aglycone is hindered by its branched-chain structure (see Kamil *et al.* 1953*a*, *b*). Consequently, the only structural requirement for a glucuronyl acceptor in the reaction studied would seem to be the availability of a stable alcoholic or carboxylic hydroxyl group. The report by Isselbacher & Axelrod (1955) that 17:21-dihydroxypregn-4-ene-3:11:20-trione (cortisone), unlike 3α:17α:21-trihydroxypregnane-11:20-dione (tetrahydrocortisone), is not such an acceptor might be explained either by the existence of some degree of specificity or by the operation of steric factors at C-17 and C-21 in steroids such as cortisone; the latter suggestion (privately communicated by Dr I. D. E. Storey) is supported by the evidence of Edwards & Kellie (1956) that glucuronic acid is conjugated *in vivo*

Table 4. *Inhibition of o-aminophenylglucuronide synthesis*

Each flask received substrates as indicated, with enzyme source: *A*, washed microsomes from 40 mg. wet wt. of liver; *B*, total homogenate from 10 mg. wet wt. of liver. Other additions and incubation as in Table 3.

Substrate (10 mM)	*o*-Aminophenol conjugated (μg.)		Inhibition (%)	
	A	*B*	*A*	*B*
o-Aminophenol, 0·7	2·1	1·7	—	—
o-Aminophenol, 0·7 + benzoate, 1·5	1·1	1·0	48	41
o-Aminophenol, 1·4 + benzoate, 1·5	1·8	1·4	15	18
o-Aminophenol, 1·4 + benzoate, 3·0	—	1·2	—	29
o-Aminophenol, 0·7	—	2·6	—	—
o-Aminophenol, 0·7 + α-ethylhexanoate, 1·4	—	1·9	—	27
o-Aminophenol, 0·9 + α-ethylhexanoate, 1·4	—	2·1	—	19
o-Aminophenol, 1·1 + α-ethylhexanoate, 1·4	—	2·3	—	12
o-Aminophenol, 1·3 + α-ethylhexanoate, 1·4	—	2·4	—	8

with the C-17 atom of androst-4-en-17β-ol-3-one (testosterone).

Naturally occurring glucuronides are considered to be of β-configuration, and all those studied in this work were found to have this structure. As already pointed out (Leloir, 1956; Storey & Dutton, 1956), there is indirect evidence that the glucuronic acid–phosphate linkage in UDPglucuronic acid is of α-configuration; the nucleotide has been obtained by enzymic oxidation of the probably α-linked uridine diphosphate glucose (Strominger *et al.* 1954), and it does not itself appear to be a substrate for β-glucuronidase (Dutton & Storey, 1954). An inversion would thus seem to occur during transfer of glucuronic acid from the nucleotide to the acceptor.

Most of the earlier work on glucuronide synthesis *in vitro* used aglycones such as a phenol (e.g. *o*- or *m*-aminophenol) or an alicylic alcohol (e.g. menthol or borneol); although such 'foreign' substances when fed to the animal are excreted as glucuronides, it has been objected (see Fishman, 1955) that they are not 'natural' substrates for the glucuronide-synthesizing system, as would be, presumably, hydroxylated steroids. The present work shows that examples of both 'foreign' and 'natural' glucuronidogenic substances accept glucuronic acid from UDPglucuronic acid, and that so far as reaction with the glucuronyl donor is concerned, no distinction exists.

Though detailed study will require a stable enzyme solution there seems no reason to suppose that the enzyme system responsible for glucuronyl transference from UDPglucuronic acid differs among these various acceptors or that it requires the participation of β-glucuronidase. So far the system has been demonstrated only in liver microsomes; there are indications that it may be present to a small extent in the kidney, slices of which organ synthesize *o*-aminophenyl glucuronide (Storey, 1950). In spite of the report by Shirai & Ohkubo (1954*b*) that rat-duodenum slices readily form OABG, the enzyme system was not found in homogenates of this organ from the mouse.

SUMMARY

1. Evidence is presented, from chromatographic and enzymic methods, for the synthesis in mouse-liver preparations of the glucuronides of *o*- and *p*-aminobenzoic acids, α-ethylhexanoic acid, 1:1-dimethylpropanol and pregnane-3α:20α-diol. Glucuronide formation was also observed when 3α-hydroxyandrostan-17-one (androsterone), *allo*pregnane-3β:20β-diol or 4-4′-dihydroxy-$\alpha\beta$-diethylstilbene was added to the preparations.

2. The presence of the glucuronyl donor uridine diphosphate glucuronic acid was necessary in all cases.

3. After an 'ester' glucuronide had been formed by such transfer of glucuronic acid, uridine 5′-pyrophosphate was demonstrated chromatographically in the reaction mixture.

4. The enzyme system responsible was located chiefly in the microsomal fraction of a mouse-liver homogenate. The specificity of this enzyme system for the various acceptors is discussed.

5. It is concluded that uridine diphosphate glucuronic acid acts as glucuronyl donor in the formation of 'ether' and 'ester' glucuronides of a wide range of compounds, including both 'foreign' and 'natural' glucuronidogenic substances.

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Purification of Bradykinin by Ion-Exchange Chromatography*

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Bradykinin, as prepared by incubation of plasma globulins with the venom of *Bothrops jararaca* (Rocha e Silva, Beraldo & Rosenfeld, 1949), can be assayed upon the guinea-pig ileum, producing a typical contraction of the slow type, not influenced by atropine or anti-histamines. From the crude material, bradykinin is quantitatively extracted into acetic acid, from which it can be precipitated by addition of several volumes of ethyl ether (Prado, Beraldo & Rocha e Silva, 1950). The dry material so obtained is completely soluble in water and can be used as a starting material for further purification. This has been achieved by employing a column of aluminium oxide (Prado *et al.* 1950) and a cellulose (paper pulp) column, according to Helmer (1950), or both (Andrade, Diniz & Rocha e Silva, 1953). We have shown that a combination of the cellulose column and aluminium oxide affords an efficient means of increasing activity of the material, from 4 to 5 'units' per mg. to 1200 'units' per mg.

For reasons given in a previous paper (Andrade, *et al.* 1953), we concluded that bradykinin is a polypeptide; electrophoresis (C. R. Diniz, personal communication) indicated that it has a high isoelectric point (about pH 10) and therefore the application of the carboxylic resin IRC-50 (XE-64) was indicated for further purification, as reported in a preliminary communication (Andrade, 1954).

EXPERIMENTAL

Crude bradykinin preparations. Large batches of ox plasma were precipitated at room temperature with an equal volume of a 50% solution of ammonium sulphate, the precipitate collected in the centrifuge was resuspended in distilled water and dialysed against running tap water for 36–48 hr. The concentrated solution of the globulins so obtained was adjusted to the same volume as that of the original plasma and incubated with venom in 500 ml. lots as described previously (Rocha e Silva *et al.* 1949). The active material was extracted with boiling 70% ethanol and dried. The dried residue was dissolved in a minimum of acetic acid and precipitated with 8 vol. of ethyl ether. The powder was dried over ether and acetone (Prado *et al.* 1950), giving a strongly yellow material, very stable at room temperature. At this stage, about 0·2 mg. of material (equivalent to 1 unit) was obtained from each millilitre of plasma.

Paper-pulp material. About 1·0–1·5 g. of the raw material obtained by precipitation from acetic acid, with an approximate activity of 4–5 'units' per mg., was dissolved in 10 ml. of water added to the top of a column of Whatman no. 1 paper pulp (Helmer, 1950) and 1 ml. of a 80% (w/v) solution of phenol was immediately added with gentle stirring. The details of the procedure for elution and freeze-drying of the active material have been given by Andrade *et al.* (1953). The dry material so obtained has also been used as a standard (PPM-*paper pulp material*) with an activity of 10 'units' per mg. The material so obtained is still yellowish, with a lustrous appearance, and is stored in the ice-box under vacuum in sealed ampoules.

Paper pulp–aluminium oxide material. In a typical experiment, 500 mg. of the dry 'paper pulp material' was dissolved in 5 ml. of 70% (v/v) ethanol and placed in the top of a column (2·5 cm. × 10 cm.) of Brockmann's aluminium oxide (Merck and Co., Rahway, N.J.). About 200 ml. of 70% ethanol was used as the eluting solution. Samples of 1 ml. were collected at about 25 min. intervals with an automatic fraction collector (Technicon, U.S.A.). The samples were analysed for biological activity, for light-absorption at 270 mμ. and for ninhydrin-reacting material, before and after hydrolysis. At this stage the non-hydrolysed material gave consistently negative reactions with ninhydrin.

Amberlite IRC 50 (*XE*-64). Chromatography with this ion-exchange resin was carried out on a 0·9 cm. × 15 cm. column. The preparation of the resin and operation of the column were carried out according to the procedure of Hirs, Moore & Stein (1953). In experiments on rechromatography of the material through the Amberlite column, the eluting solution had a pH higher than that of the column and therefore a gradient of pH was established during elution. This technique has also been used for purifying

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